

WDN/SAS/DJZ:kam 04/17/02 111
PATENTAttorney Reference Number 4239-61854
Application Number 10/031,158

phenylalanyl-tRNA synthetase (Mosyak, L. *et al.*, *Nat. Struct. Biol.* 2:537-547 (1995)) have shown that these proteins do not contain α -helical structures in the region where the sequence contains five leucines in heptad repeats. Interaction and structure studies are needed to determine the significance of the leucine repeats found in TARP.--

Please replace the paragraph at Page 13, lines 1 through 8 with the following:

--Another unusual feature of the TARP amino acid sequence is that a region of basic amino acids follows the potential leucine zipper motif (Figure 14A), suggesting a possible DNA-binding motif. However, the orientation of the basic region is rather unique in that it follows the leucine repeats rather than precedes them. Most leucine zipper proteins that bind DNA have the basic region before the leucine repeats (for a review, see (Chook, Y. M. *et al.*, *Nature* 399:230-237 (1999))). The basic region in TARP may only be functioning as a nuclear localization signal, but the fact that TARP is a nuclear protein strengthens the hypothesis that TARP may bind DNA.--

Please replace the paragraph at Page 13, lines 9 through 28 with the following:

--To determine if TARP shares homology with any known proteins, we performed a protein BLAST search against GenBank. This search indicated that the amino acid sequence of TARP shares some homology to *Dictyostelium discoideum* Tup1 (GenBank accession no. AAC29438) and *Saccharomyces cerevisiae* Tup1 (Williams, F. E. *et al.*, *Mol. Cell. Biol.* 10:6500-6511 (1990)) (Figure 14E). Yeast Tup1 is normally found in a complex with Cyc8(Ssn6) and is required for transcriptional repression of genes that are regulated by glucose, oxygen and DNA damage (Tzamarias, D. *et al.*, *Genes Dev.* 9:821-831 (1995)). Neither Cyc8(Ssn6) nor Tup1 binds DNA, but each acts as a part of a corepressor complex through interactions with specific DNA-binding proteins such as α 2, Mig1, Rox1 and a1 (Tzamarias, D. *et al.*, *Genes Dev.* 9:821-831 (1995)). The C'-terminal half of Tup1 contains six repeats of a 43-amino acid sequence rich in aspartate and tryptophan, known as WD-40 or β -transducin repeats (Williams, F. E. *et al.*, *Mol. Cell. Biol.* 10:6500-6511 (1990); Fong, H. K. *et al.*, *Proc. Natl. Acad. Sci. USA* 83:2162-2166 (1986)). WD-40 repeats have been identified in many proteins and play a role in protein-protein interactions. Importantly, Tup1 has been shown to interact with α 2 through two of its WD-40 repeats (Komachi, K. *et al.*, *Genes Dev.* 8:2857-2867 (1994)).

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It is interesting to note that TARP shares homology with the fifth WD-40 repeat of Tup1 (Figure 7C). Because TARP is a nuclear protein, its homology with Tup1 suggests that TARP is a member of a functional nuclear protein complex involved in transcriptional regulation.--

Please replace the paragraph at Page 13, lines 29 through 34, and Page 14, lines 1 through 10 with the following:

--The TARP antibody recognizes a doublet in prostate and breast nuclear extracts (Figure 13A). The faster 7 kDa band comigrates with the His-TARP recombinant protein, while the weaker band runs at a larger molecular weight. One possible explanation for the 9 kDa band is post-translational modifications. To determine if TARP contains any known post-translational modification sites, we analyzed the TARP amino acid sequence using the PROSITE program of the *Swiss Institute of Bioinformatics* ExPASy proteomics server (Appel, R. D. *et al.*, *Trends Biochem. Sci.* 19:248-260 (1994); Hofmann, K. *et al.*, *Nucleic Acids Res.* 27:215-219 (1999)). As shown in Figure 14A, many potential phosphorylation sites were found including cAMP- and cGMP-dependent protein kinase phosphorylation sites (RRAT and RRGTT) and protein kinase C phosphorylation sites (SSR and SRR). Phosphorylation has been shown in many cases to cause a protein to run at a larger apparent molecular weight on an SDS-PAGE gel. If this is the case, the results from Figure 13 may indicate that the unmodified form is prevalent in LNCaP cells and that only the phosphorylated form is present in MCF7 and SK-BR-3 cells. TARP may therefore be post-translationally modified when expressed in prostate and breast cancer cells.--

Please replace the paragraph at Page 14, lines 33 and 34, and Page 15, lines 1 through 9 with the following:

--The prostate is dependent on androgens for maintenance of its structure and function. When prostate cells become malignant, they often lose their androgen dependence. In this study, we used two prostate cell lines that differ in their dependence on androgen for growth: LNCaP and PC3 cells. The androgen receptor is present in the androgen-dependent LNCaP cell line, but is absent in the androgen-independent PC3 cell line (Tilley, W. D. *et al.*, *Cancer Res.* 50:5382-5386 (1990)). As shown in Figure 10, *TARP* is expressed in LNCaP cells but not in PC3 cells. This result suggests that *TARP* expression may be regulated by androgen stimulation. The identification of an ARE-like element within the *TARP* promoter strengthens the idea that *TARP*

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is induced by androgens. Expression in LNCaP cell but not in PC3 cells suggests that *TARP* is important in regulating androgen-dependent responses.--

Please replace the paragraph at Page 15, lines 24 through 29 with the following:

--"T-cell receptor γ Alternate Reading frame Protein" and "TARP" refer to the polypeptide whose sequence is set forth, e.g., in Figure 1. The polypeptide is translated from a form of the T-cell receptor γ gene present as a transcript in prostate cells of epithelial origin, in prostate cancer cells, and in many breast cancers. Since "TARP" is an acronym the last part of which stands for the word "protein," "TARP protein" is redundant.--

Please replace the paragraph at Page 23, lines 25 through 32, and Page 24, lines 1 and 2 with the following:

--This invention provides isolated, recombinant TARP. Because we first found isolated a prostate-specific TCR γ transcript, we initially used the terms "PS-TCR γ protein" and "PS-TCR γ polypeptide" to refer to any polypeptide that could be translated in any reading frame from the ~1.1 kb PS-TCR γ transcript. In particular, the terms referred to two proteins, PS-TCR γ -1 (SEQ ID NO:14) and PS-TCR γ -2 (SEQ ID NO:15), translated in *in vitro* translation systems. We have now determined that only the first of these reading frames is translated in prostate cells. Since this reading frame is not the reading frame which results in the TCR γ chain, the protein is now referred to as the "T-cell receptor Alternate Reading frame Protein." Full-length TARP is a 58 amino acid protein whose sequence is set forth in SEQ ID NO:14 and Figure 1.--

Please replace the paragraph at Page 25, lines 1 through 8 with the following:

--In one aspect this invention provides an isolated, recombinant nucleic acid molecule comprising a nucleotide sequence encoding the TARP polypeptide (see, e.g., Figure 1). This nucleic acid is useful for expressing TARP, which can then be used, for example, to raise antibodies for diagnostic purposes. As noted, the nucleic acid molecule has three reading frames, each of which encodes different polypeptides defined by different open reading frames. In the embodiments contemplated herein, the reading frame of interest is the one which encodes TARP.--

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Please replace the paragraph at Page 55, lines 19 through 32, and Page 56, lines 1 and 2 with the following:

--TARP is a nuclear protein expressed in prostate cancer cells. To determine whether TARP or TCR γ exists in prostate cancer cells, we generated antibodies against both proteins and performed western blots on different prostate cancer cell extracts. As shown in Figure 10A (top panel), TARP was detected in the prostate cancer LNCaP cell line and a prostate cancer tumor extract. The 7 kDa band comigrates with the recombinant His-TARP suggesting that the product detected in the LNCaP and cancer extracts is TARP. Previously, we demonstrated that the prostate-specific TCR γ transcript is not expressed in the prostate cancer PC3 cell line (Essand, M. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:9287-9292 (1999)). Therefore, we used PC3 cell extracts as a negative control and demonstrated that the 7 kDa band was absent in these extracts (Figure 10A, top panel). Importantly, no 7 kDa bands were detected when the pre-bleed antiserum or an antiserum against the *Pseudomonas* exotoxin (PE, see Materials and Methods) was used (data not shown). TCR γ was not detected in any of these extracts even though the recombinant protein showed a very strong signal with the antibody employed (Figure 10A, bottom panel). These data indicate that the prostate-specific TCR γ transcript encodes TARP.--

Please replace the paragraph at Page 56, lines 3 through 7 with the following:

--To determine the cellular localization of TARP, we prepared nuclear, cytoplasmic and membrane fractions from LNCaP cells. As shown in Figure 10B, TARP was detected in the nucleus and not in the cytoplasm or membrane fraction. Similar results were obtained using nuclei purified by fractionating the cell extracts through a sucrose cushion (Sladek, F. M. *et al.*, *Genes Dev.* 4:2353-2365 (1990)) (data not shown).--

Please replace the paragraph at Page 56, lines 8 through 32 with the following:

--The TARP transcript is expressed in breast cells. Previously, we reported that the TCR γ EST cluster also contains some ESTs from brain libraries (Vasmatazis, G. *et al.*, *Proc. Natl. Acad. Sci. USA* 95:300-304 (1998)). After this initial report, additional ESTs have been deposited into the database and the cluster now contains ESTs from breast, colon, kidney and gastric libraries as well. To determine whether the existence of these ESTs indicates the

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expression of the *TARP* transcript in these cells or whether it may be due to the presence of infiltrating $\gamma\delta$ T-lymphocytes when these libraries were made, we performed RT-PCR on various cell lines to test for the presence of the *TARP* transcript. As shown in Figure 11A, expression of the *TARP* transcript was detected in the breast cell lines MCF7, BT-474, SK-BR-3 and CRL-1897. No signals were detected in the neuroblastoma cell line A172, glioblastoma cell line IMR32, colon cell line COLO 205, gastric cell line KATO III or kidney cell lines COS7 and 293 (Figure 11A and data not shown). To determine whether the *TARP* transcript is expressed in human breast tissues in addition to cell lines, we tested 12 different normal breast and 12 different breast cancer cDNAs using a RAPID-SCANTM panel (OriGene Technologies, Rockville, MD). *TARP* mRNA was shown to be abundant in some of the breast cancer samples (Figure 11B, top panel) while barely detectable in the normal breast samples after 35 rounds of PCR (data not shown). Significantly, no signals were detected in reactions lacking cDNA. *Actin* was used to show that similar amounts of cDNA were present in each lane (bottom panel). The weak signals in the normal breast samples correlate well with the lack of *TARP* signal shown in Figures 11A and 12 for the Hs57Bst cell line, a breast cell line derived from normal breast tissue. These results suggest that expression of the *TARP* transcript in the breast is increased after oncogenic transformation. However, more studies are needed before any definitive conclusions can be made.--

Please replace the paragraph at Page 57, lines 1 through 20 with the following:

--To determine whether the *TARP* transcript observed in the breast cell lines is the same as the transcript found in the prostate cell line, we performed RT-PCR using primers against different regions of the *TARP* transcript. As shown in Figure 12A, the *TARP* transcript in prostate contains a portion of the Jy1.2 gene segment, three Cyl exons and some untranslated sequence followed by a poly(A) tail (7). Primer set 1 and 3 amplifies the entire *TARP* transcript (Figure 12B, top panel) while primer set 2 and 3 amplifies the Cyl region only (Figure 12B, middle panel). As shown in Figure 12B, similar-sized bands were detected in three breast cell lines (MCF7, BT-474 and SK-BR-3) as compared to the prostate cell line (LNCaP) using either primer set. Importantly, no signals were detected in the reactions lacking cDNA (dH₂O) and similar amounts of cDNA were used as demonstrated by the *actin* control (Figure 12B, bottom panel). These data indicate that the *TARP* transcript found in the breast cell lines is the same as

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the transcript found in the prostate cell line. To further support this conclusion, we analyzed the *TARP* transcript sizes from each cell line by a northern blot. Previously, we showed that 1100 and 2800 nucleotide transcripts exist in LNCaP cells, with the 1100 nucleotide transcript being the predominant form (Essand, M. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:9287-9292 (1999)). As shown in Figure 12C, similar-sized *TARP* transcripts were found in three breast cell lines (MCF7, BT-474 and SK-BR-3) as compared to the prostate cell line (LNCaP), although at a weaker intensity. Therefore, we conclude that *TARP* mRNA is expressed in prostate and breast cancer cells.--

Please replace the paragraph at Page 57, lines 21 through 29 with the following:

--To determine whether TARP protein exists in the breast cancer cell lines, we performed a western blot with breast cancer nuclear extracts using an antibody against TARP. As shown in Figure 13 (top panel), TARP reactive bands were detected in MCF7, BT-474 and SK-BR-3 cells. TARP was not detected in the membrane or cytoplasmic fractions in these breast cancer lines (data not shown). Importantly, TARP is the protein product encoded by the *TARP* transcript in the breast cell lines because TCR γ was not detected in any of these nuclear extracts even though the recombinant protein showed a very strong signal with the antibody employed (Figure 13, bottom panel). These data indicate that TARP also exists in breast cancer cells.--

Please replace the paragraph at Page 57, lines 30 through 33, and Page 58, lines 1 through 10 with the following:

--We report the identification of a 7 kDa nuclear protein encoded by a specific transcript derived from the *TCR γ* locus expressed in prostate and breast cancer cells. Because the protein is encoded from a reading frame different from TCR γ , we name it TARP for TCR γ Alternate Reading frame Protein. Besides being translated from an alternate reading frame of a transcript originating within an intron of the *TCR γ* locus, TARP has two other unusual features. First, it is surprising to find such a small peptide in the cell because most are usually secreted. Second, TARP lacks a good Kozak sequence (Kozak, M. *Cell* 44:283-92 (1986)). Because the TCR γ reading frame contains a good Kozak sequence, we initially hypothesized that a truncated TCR γ protein was encoded. However, as shown in Figure 10, our initial hypothesis was incorrect. It is

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of interest that the *in vitro* translation results indicate a preference for the TARP protein and that either ATG in the TARP reading frame can be used to initiate protein synthesis. Protein sequencing will be needed to determine which ATG is used to initiate TARP protein synthesis.--

Please replace the paragraph at Page 58, lines 11 through 27 with the following:

--A very interesting feature of the TARP protein sequence is that it contains five leucines in heptad repeats, suggesting that TARP may contain a leucine zipper dimerization motif (Figure 14A). For this to be true, TARP must contain an amphipathic helix. One indication that TARP may contain an amphipathic helix is that serine and proline residues, residues believed to serve as a helix initiator, are found immediately before the first leucine repeat. Second, many charged amino acids are found within the heptad repeats thereby giving the helix an amphipathic nature and potentially serving as salt bridges with other helices. Even though the presence of leucines in heptad repeats is a good indication of a leucine zipper motif, there are proteins identified containing five leucines in heptad repeats that are not considered leucine zipper proteins. For example, the crystal structures for karyopherin (Chook, Y. M. *et al.*, *Nature* 399:230-237 (1999)), *B. stearothermophilus* pyrimidine nucleoside phosphorylase (Pugmire, M. J. *et al.*, *Structure* 6:1467-1479 (1998)) and *T. thermophilus* phenylalanyl-tRNA synthetase (Mosyak, L. *et al.*, *Nat. Struct. Biol.* 2:537-547 (1995)) have shown that these proteins do not contain α -helical structures in the region where the sequence contains five leucines in heptad repeats. Interaction and structure studies are needed to determine the significance of the leucine repeats found in TARP.--

Please replace the paragraph at Page 58, lines 28 through 33, and Page 59, lines 1 through 3 with the following:

--Another unusual feature of the TARP amino acid sequence is that a region of basic amino acids follows the potential leucine zipper motif (Figure 14A), suggesting a possible DNA-binding motif. However, the orientation of the basic region is rather unique in that it follows the leucine repeats rather than precedes them. Most leucine zipper proteins that bind DNA have the basic region before the leucine repeats (for a review, see (Chook, Y. M. *et al.*, *Nature* 399:230-237 (1999))). The basic region in TARP may only be functioning as a nuclear localization

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signal, but the fact that TARP is a nuclear protein strengthens the hypothesis that TARP may bind DNA. Functional studies are needed before any definitive conclusions can be made.--

Please replace the paragraph at Page 59, lines 4 through 24 with the following:

--To determine if TARP shares homology with any known proteins, we performed a protein BLAST search against GenBank. This search indicated that the amino acid sequence of TARP shares some homology to *Dictyostelium discoideum* Tup1 (GenBank accession no. AAC29438) and *Saccharomyces cerevisiae* Tup1 (Williams, F. E. *et al.*, *Mol. Cell. Biol.* 10:6500-6511 (1990)) (Figure 14B). Yeast Tup1 is normally found in a complex with Cyc8(Ssn6) and is required for transcriptional repression of genes that are regulated by glucose, oxygen and DNA damage (Tzamarias, D. *et al.*, *Genes Dev.* 9:821-831 (1995)). Neither Cyc8(Ssn6) nor Tup1 binds DNA, but each acts as a part of a corepressor complex through interactions with specific DNA-binding proteins such as $\alpha 2$, Mig1, Rox1 and a1 (Tzamarias, D. *et al.*, *Genes Dev.* 9:821-831 (1995)). The C'-terminal half of Tup1 contains six repeats of a 43-amino acid sequence rich in aspartate and tryptophan, known as WD-40 or β -transducin repeats (Williams, F. E. *et al.*, *Mol. Cell. Biol.* 10:6500-6511 (1990); Fong, H. K. *et al.*, *Proc. Natl. Acad. Sci. USA* 83:2162-2166 (1986)). WD-40 repeats have been identified in many proteins and play a role in protein-protein interactions. Importantly, Tup1 has been shown to interact with $\alpha 2$ through two of its WD-40 repeats (Komachi, K. *et al.*, *Genes Dev.* 8:2857-2867 (1994)). It is interesting to note that TARP shares homology with the fifth WD-40 repeat of Tup1 (Figure 14B). Because TARP is a nuclear protein, its homology with Tup1 suggests that TARP may be a member of a functional nuclear protein complex involved in transcriptional regulation. Therefore, it is necessary to identify TARP-interacting proteins in order to determine its function.--

Please replace the paragraph at Page 59, lines 25 through 34, and Page 60, lines 1 through 7 with the following:

--The TARP antibody recognizes a doublet in prostate and breast nuclear extracts (Figure 13A). The faster 7 kDa band comigrates with the His-TARP recombinant protein, while the weaker band runs at a larger molecular weight. One possible explanation for the 9 kDa band is post-translational modifications. To determine if TARP contains any known post-translational